Judy A. Spitzer, Ph.D.

NMRDC

STATUS REPORT
N00014-89-J-1916

FOR NOVEMBER 1, 1990 TO FEBRUARY 28, 1991

Investigations continued in the following areas:

1. Characterization of TNF-induced modulation of host defense mechanisms in rat alveolar macrophages

A. TNF time course

Our previous work with alveolar macrophages (AMs) and TNF treat ment utilized the 3 hr infusion or the bolus injection, 90 min sacrifice models. A time course study was undertaken in which animals were sacrificed at 30 min 1, 1.5, 2, 3, 6, 12, 24 and 48 hr after bolus injection (penile vein) of 6 X 10^5 units of human recombinant TNF in 0.5 ml. At each time point, spontaneous, PMA-stimulated and opsonized zymosan- (OPZ) stimulated release of superoxide anion (SO) was measured in AMs obtained by broncho-alveolar lavage. In addition, TNF levels were measured in the sera of all of the TNF-injected rats (courtesy of Dr. Gregory J. Bagby of the Department of Physiology) and peripheral blood differential counts were also performed. This series of experiments demonstrated that TNF was detectable in serum up to 6 hr after a bolus injection, demonstrating rapid disappearance in the first two hr.(Fig. 1).

Peripheral blood differential counts over a 48 hr period following the initial bolus injection of TNF revealed reciprocal changes in polymorphonuclear leukocytes (PMN) and lymphocytes in TNF-treated rats. The percent distribution of the two cell types returned to normal by 48 hr (Fig. 2).

Comparison of the time course of SO release by AMs of TNF versus saline-treated rats upon OPZ (Fig. 3) or PMA (Fig. 4) stimulation revealed no significant difference due to treatment with TNF. Since in the course of our previous investigations we were able to demonstrate a significant increase in SO release upon PMA-stimulation of AMs following TNF injection, we needed to ascertain the reason for this discrepancy. As can be observed in Figure 4, SO release upon PMA stimulation showed a high degree of variability. Analysis of the spontaneous SO release values from the Sprague-Dawley rats used in more recent studies indicated a great increase and variability in this parameter during the time period when the time course studies were completed. In order to understand the reasons for the differences in results obtained and to eliminate the elevated spontaneous release of SO, a cold procedure (0-4°C) for lavaging and processing of the AMs was adopted. The cold procedure indeed resulted in much improved spontaneous release of SO. AMs of only 1 out of 18 rats showed a level of spontaneous release

comparable to what was observed previously with the warm procedure (Fig. 5). However, PMA-stimulated SO release of saline-treated rats was still very variable. The cold procedure seemed to produce a less variable response to OPZ stimulation in the saline-treated animals than what was obtained with PMA stimulation.

B. TNF dose response studies

Rats received a bolus injection in a total volume of 0.5 ml into the penile vein, 90 min prior to sacrifice. The concentrations of TNF used were 6 X 10^5, 1.15 X 10^6, and 2.3 X 10^6 units. The cold procedure was used up until the time of incubation in the presence of the various agonists. The animals were barrier-raised Sprague-Dawley rats obtained from Charles River Breeding Laboratories and spontaneous, PMA (10^{-8}M) and OPZ- (2000 ug/10^6 cells) stimulated SO release was measured. In these experiments also, the PMA-induced SO release showed great variability. Values for saline-treated rats ranged from 3.9 to 82.7 nmoles/10^6 cells/30 min. Thus, it was not possible to conclusively evaluate the effect of TNF injection on this parameter. The OPZ response was much less variable, and higher overall than the response to PMA.

In order to assure the reproducibility of our initial studies, we have decided to try to use CVF (certified virus-free) Sprague-Dawley rats from Hilltop Lab Animals, Inc. These rats are provided with negative antibody profile to a series of bacteria, viruses and mycoplasma that can commonly infect these rats. Our preliminary experiments have shown that using these rats 24 hr after arrival leads to a PMA-induced SO release with less variability than that previously observed in the experiments referred to in this report.

These data call attention to the significance of the variability in the quality and the immune status of barrier-raised animals versus certified virus-free rats. We are currently devising ways and means whereby we can purchase CVF rats and provide appropriate facilities for them for the short term so that we can eliminate the variability that we have observed lately in barrier-raised animals.

C. Mediating mechanisms -- arachidonic acid metabolism

In order to explore some of the mechanisms mediating TNF-induced changes in AM function, we focused on important lipid mediators, namely arachidonic acid (AA) metabolites. The eicosanoid profile of naive-control rats was determined in [1^{14}C] AA-labeled AMs under basal conditions and upon stimulation with 1μM A23187. Using our standard HPLC methods, we have detected the release of both cyclooxygenase (CO) and lipoxygenase (LO) products. The distribution of the released AA metabolites indicated that CO products accounted for 30%, and LO products for 70% of the total AA metabolites released. Calculated as percent of released products, TxB_2 was the major CO product while 15-HETE and 5-HETE were the major LO products. If the data are calculated as percent increase over basal release, the major CO products are TxB_2,

$PGF_{2\alpha}$, PGE_2, PGD_2, and HHT, while the major LO products are LTB_4, and 5-HETE. This distribution agrees well with reports in the literature pertaining to AA metabolites released by AMs. With conditions set and optimized in the cells of naive control animals, we are now ready to evaluate the effect of TNF on AA metabolism in AMs following either *i.v.* or intratracheal injections.

2. Studies with alveolar macrophages obtained from endotoxin-(ET) treated rats

A. Time course study

A time course study of the effect of an *i.v.* bolus injection of a non-lethal dose of ET, on OPZ-stimulated SO release was completed. The results indicated that ET primes rat AMs for a supernormal release of SO 1.5 hr following ET injection. As can be observed from Figure 6, this effect was not present at an earlier (0.5 hr post injection) or at a later time, namely at 3 hr post-injection. Using two-way analysis of variance followed by a Student-Newman-Keuls test, the 1.5 ET group was found to be significantly different from all of the other groups. There was no difference among the saline control groups at the various times.

B. Comparative effectiveness of PMA and OPZ stimulation for SO release

The optimal PMA concentration for stimulation of AMs in our model was 10^{-8}M and 3.4 mg/10^6 cells (0.5 mg/ml) for OPZ concentration. As can be seen in Figure 7, a comparison of stimulation of rat AMs using optimal concentrations of OPZ and PMA indicates OPZ to be a more potent stimulus for SO release. One way analysis of variance followed by a Student-Newman-Keuls test indicated that there was no difference between the saline- and ET-treatment groups when PMA was the stimulant, whereas there was a significant difference between the two groups upon OPZ stimulation. OPZ-stimulated AMs from the ET-treated group exhibited a super-normal release, suggesting that these cells were already primed due to the *in vivo* ET treatment. SO release in response to OPZ stimulation was significantly higher in AMs of both saline- and ET-treated rats -- 72 % more in saline and 178.5% more in the ET-treated groups, respectively.

The role of PKC in the signal transduction mechanisms of OPZ-stim ulated SO release was investigated using the PKC inhibitor staurosporine (ST). Figure 8 presents the results of these experiments using both agonists, i.e. PMA (10^{-8}M or OPZ (3.4 mg/10^6 cells, 0.5 mg/ml) and increasing concentrations of ST. A plateau of 80% inhibition of OPZ-stimulated SO release was achieved in both the saline- and ET-treatment groups with ST concentrations between 100-200 nM. Maximal inhibition of PMA-stimulated SO release was obtained at a much lower ST concentration (25 nM). There was no difference in the degree of inhibition of PMA- or OPZ-stimulated SO release in the two treatment groups.

The results are <u>significant</u> in terms of demonstrating that *in vivo* ET treatment results in differential priming for subsequent *in vitro* stimulation of SO release. The particulate stimulus provided by

OPZ resulted in a super-normal release of SO in ET AMs, while stimulation with PMA did not result in a difference in SO release between AMs of saline- and ET-treated rats. The data are consistent with the notion that in both saline- and ET-treatment groups, OPZ stimulation is likely to involve a non-PKC mediated signal transduction mechanism(s).

3. Superoxide anion release by Kupffer and endothelial cells, and PMNs obtained from the livers of rats infused for 3 hr with a non-lethal dose of ET

SO release was determined in nonparenchymal (NP) cells of saline- and ET-infused animals. The NP cells of ET-treated rats were further fractionated by elutriation and subsequent Histopaque-Ficoll gradient separation into Kupffer cells, PMNs and endothelial cells. As can be seen in Figure 9, SO production in both OPZ- and PMA-stimulated NP cells obtained from ET-treated rats is significantly higher than in saline control animals stimulated with the same agonists. SO production by endothelial cells is minimal in both treatment groups.

Since after 3 hr of ET infusion, neutrophil sequestration in the liver is significant (Rodriquez de Turco, E.B. and J.A. Spitzer, J. Leukocyte Biology 48:488-494, 1990), it was of interest to determine the contribution of PMNs and Kupffer cells to the elevated production of SO observed. Cell distribution in the 45 ml/min elutriated fraction corresponding to the Kupffer cell fraction as well as cell distribution after the gradient separation are presented in Table 1. Table 2 presents SO production spontaneously and upon PMA and OPZ stimulation in the various cell fractions in saline- and ET-treated animals. As can be seen, most of the SO appears to come from the PMN fraction, although there is signficant contribution by the Kupffer cells themselves.

The significance of these data lies in the fact that they indicate *in vivo* priming of Kupffer cells and neutrophils sequestered in the liver due to a 3 hr infusion of a non-lethal dose of ET, that becomes apparent when the cells are challenged *in vitro* by a particulate or a soluble stimulus. The data are also consistent with the interpretation that at this fairly early timepoint upon ET exposure, protein kinase C- (PKC) mediated mechanisms contribute to a far greater extent than non-PKC mediated mechanisms to the enhanced respiratory burst documented in these cells.

Some of our results included in this progress report will be presented at the FASEB meeting to be held in Atlanta in April 1991. The two abstracts are attached.

Enc. 8 figures
2 tables
2 abstracts

FIG. 1.

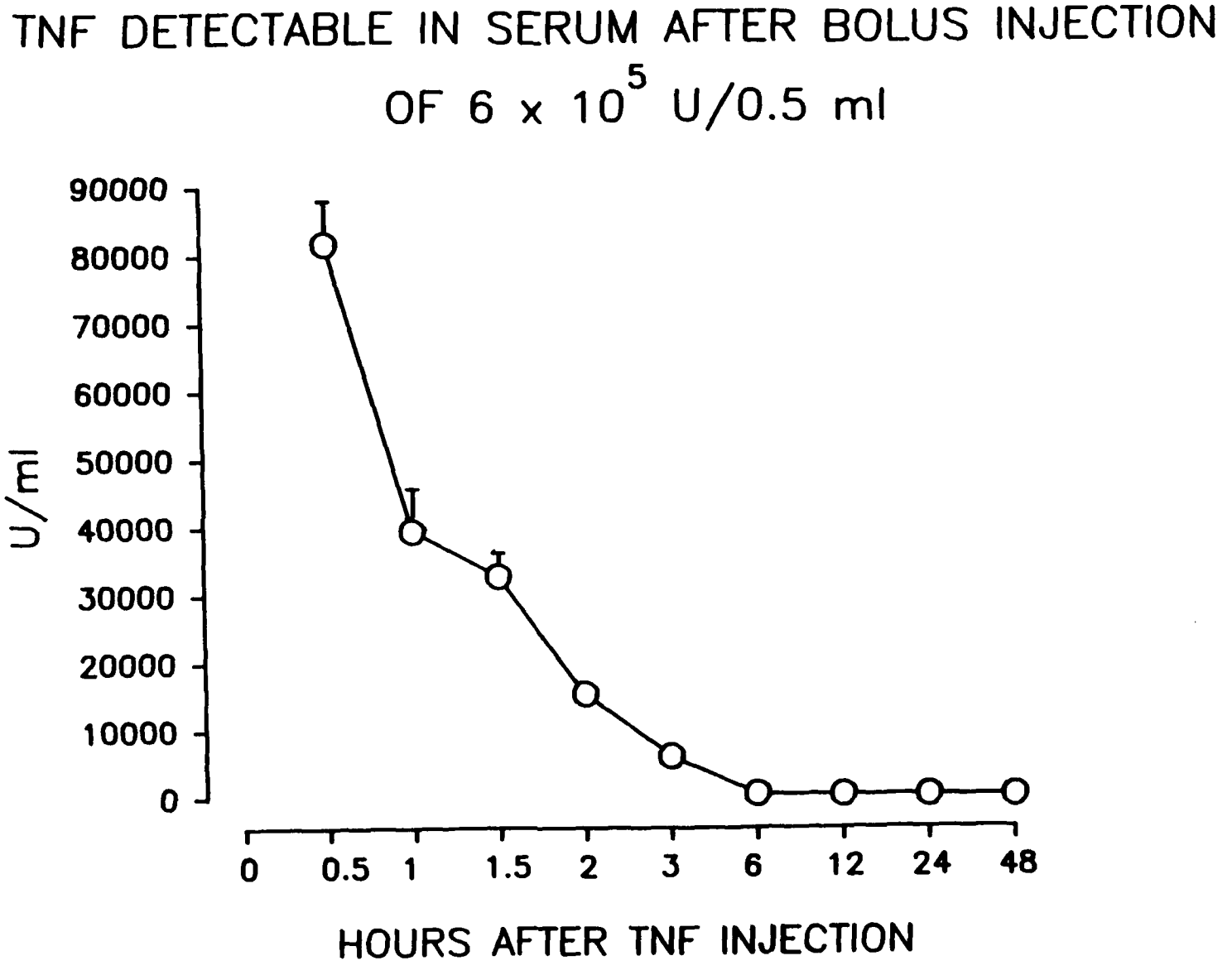
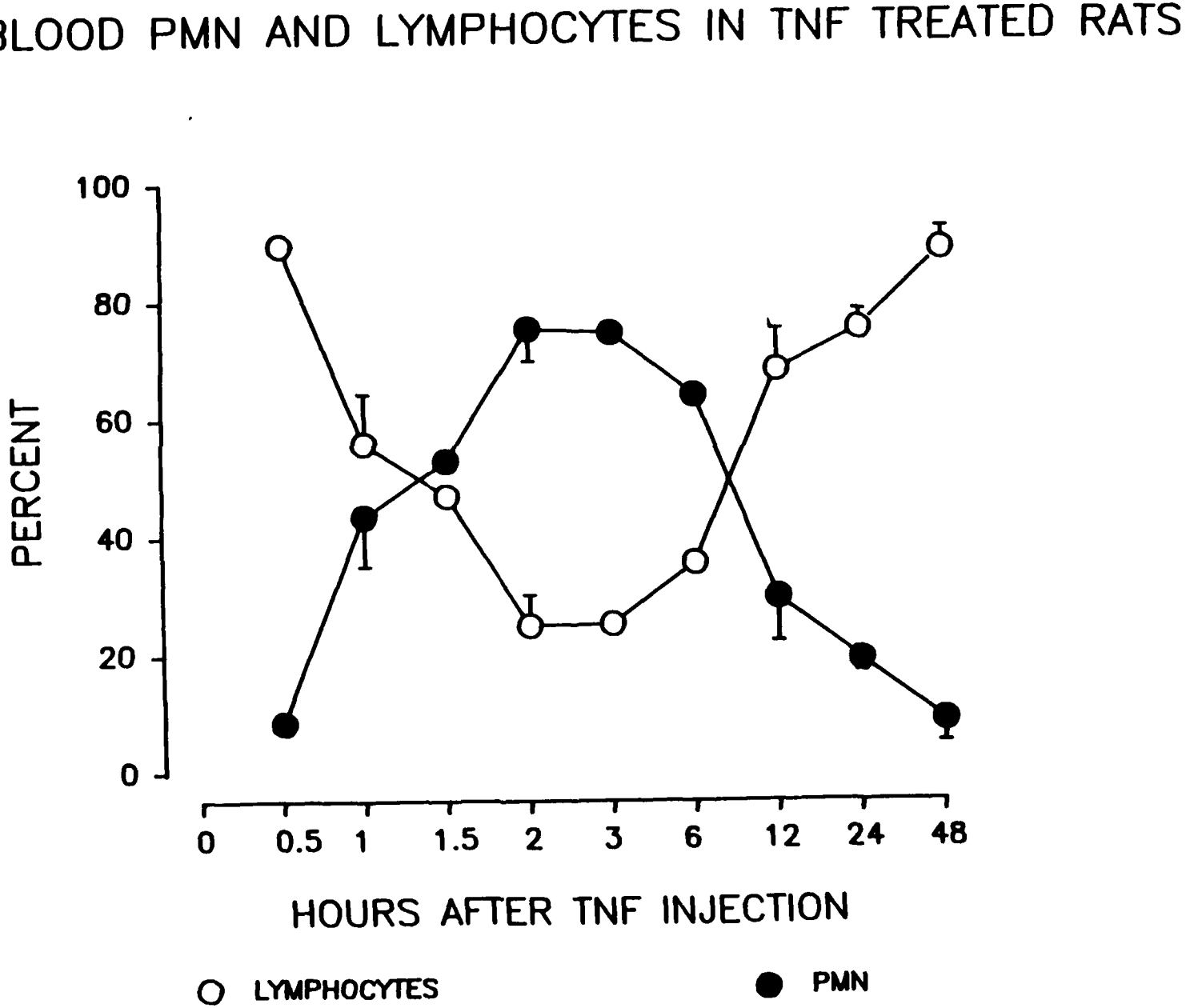

TNF DETECTABLE IN SERUM AFTER BOLUS INJECTION OF 6 x 10^5 U/0.5 ml
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
U/ml
0
0.5
1
1.5
2
3
6
12
24
48
HOURS AFTER TNF INJECTION

FIG. 2.


BLOOD PMN AND LYMPHOCYTES IN TNF TREATED RATS
PERCENT
100
80
60
40
20
0
0
0.5
1
1.5
2
3
6
12
24
48
HOURS AFTER TNF INJECTION
LYMPHOCYTES
PMN

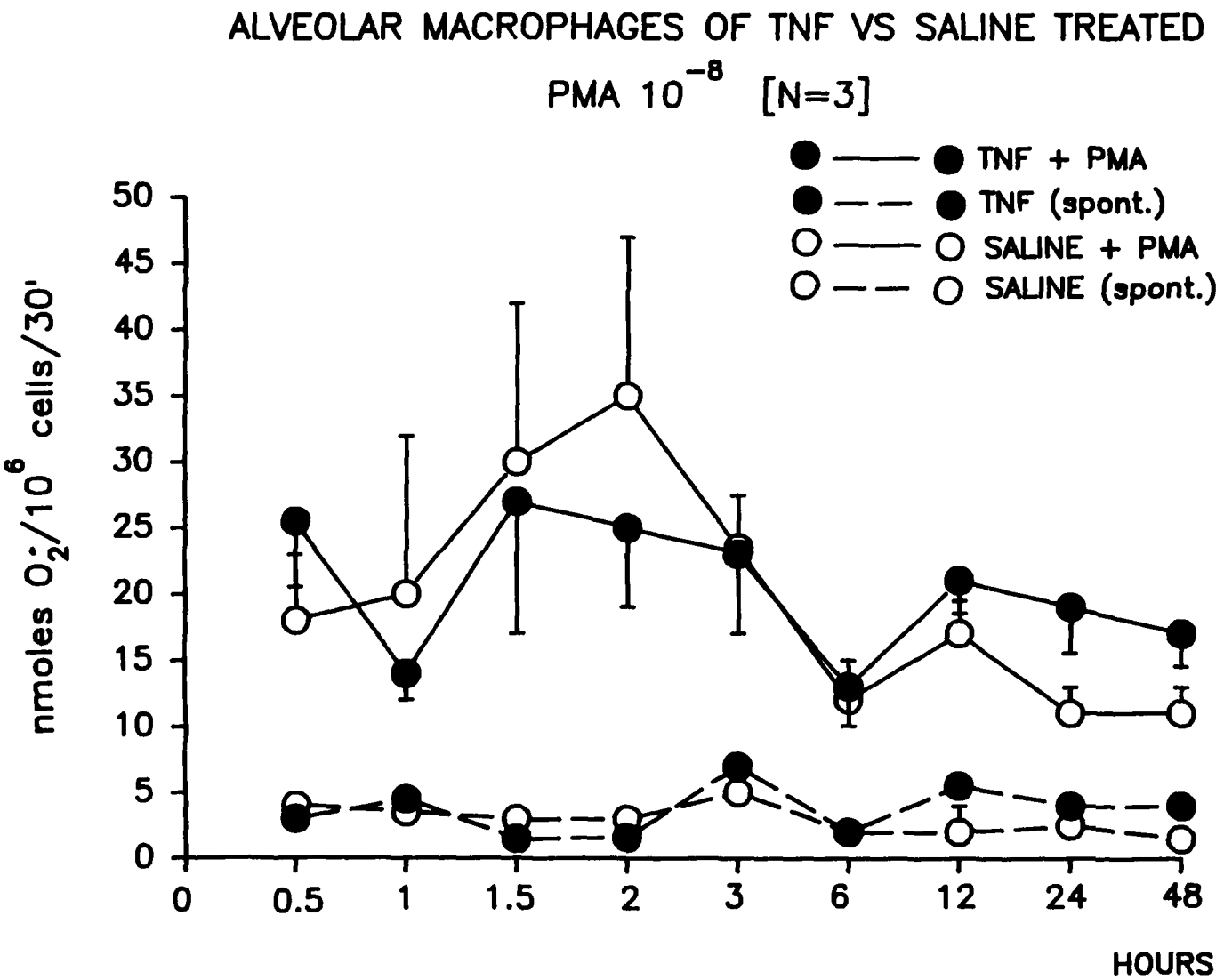


Fig. 3 Time course of PMA-stimulated SO ($O_2^{\cdot}$) release in AMs of TNF and saline treated rats.

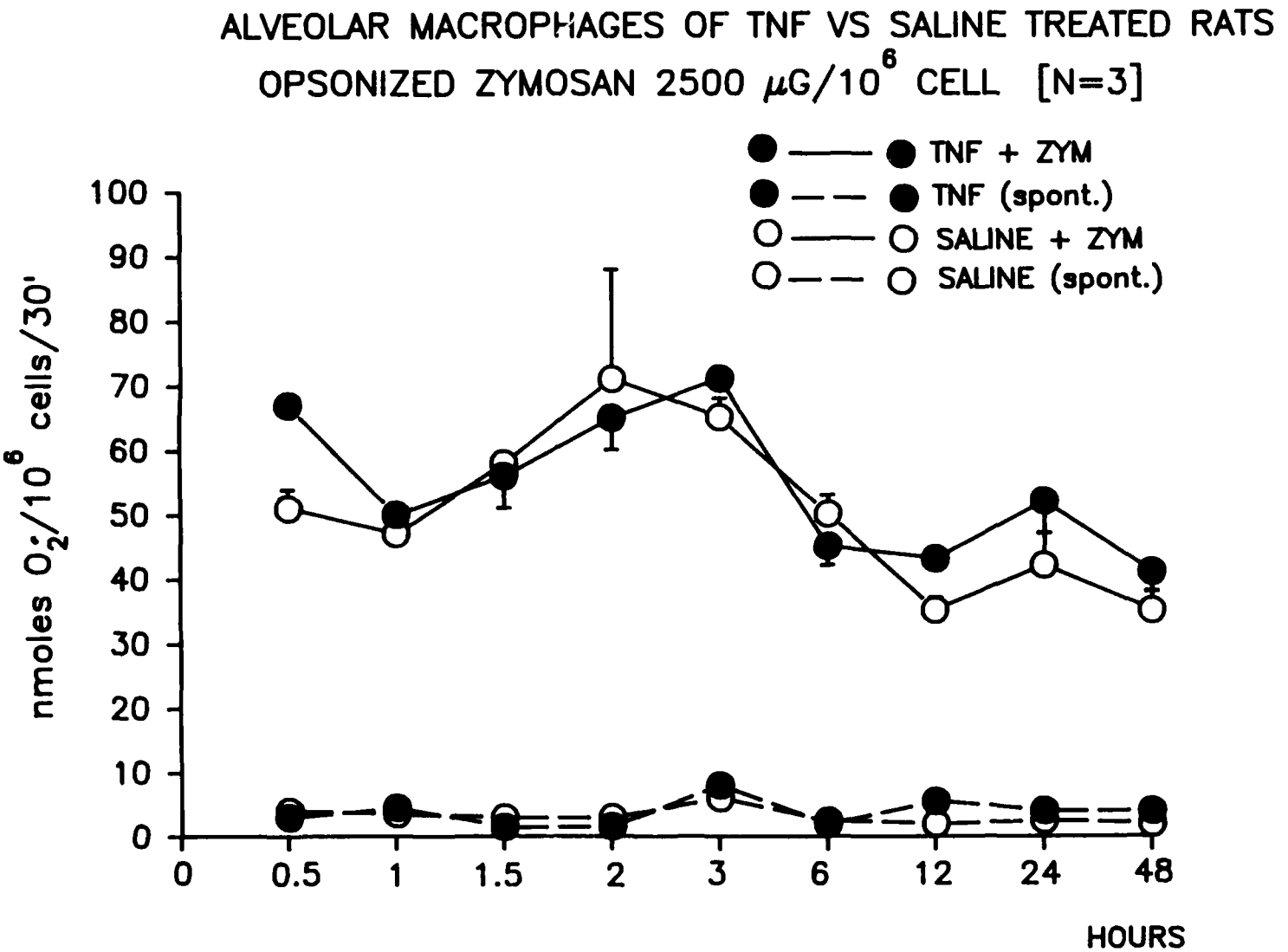
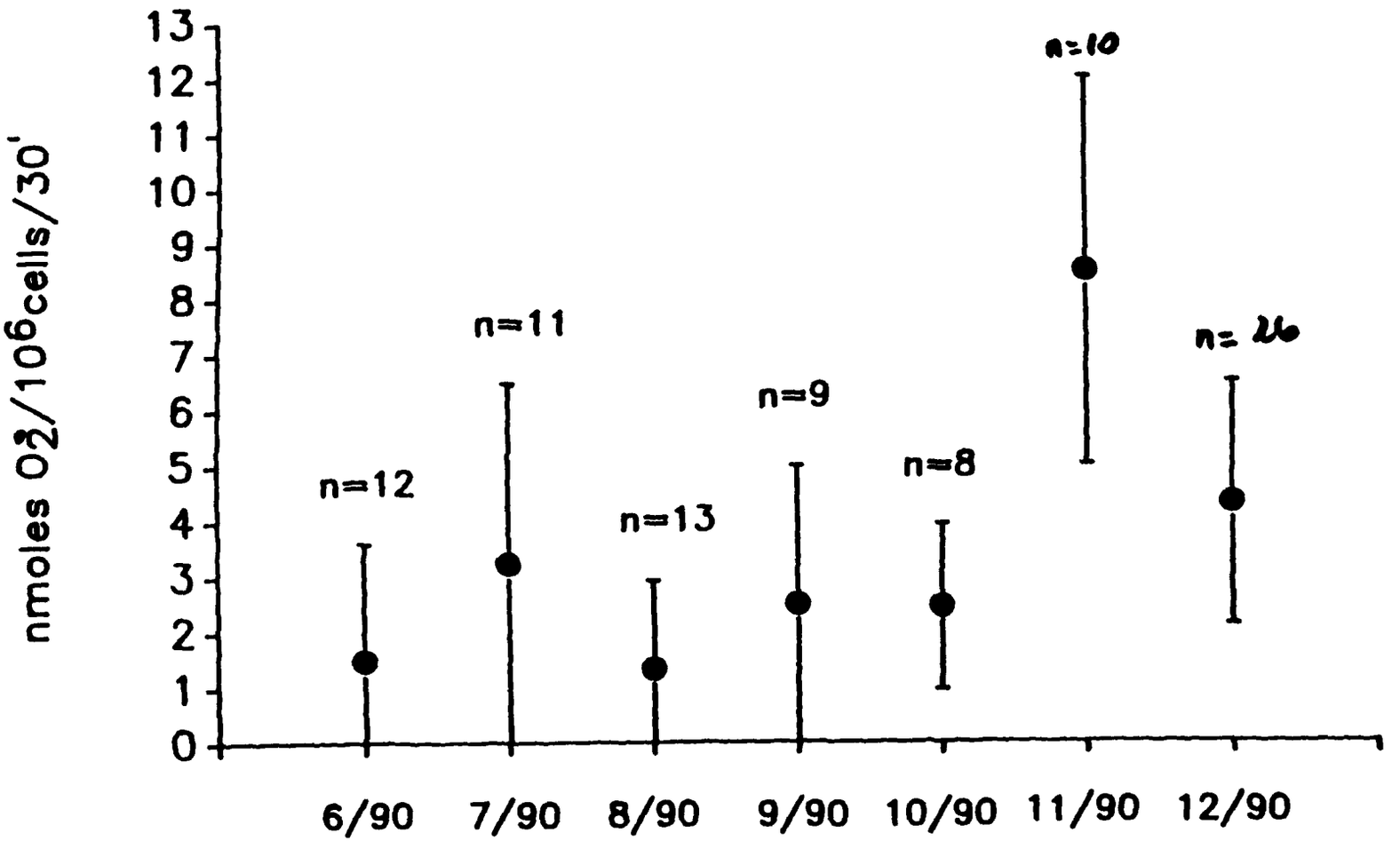


Fig. 4 Time course of OPZ-stimulated SO ($O_2^{\cdot}$) release in AMs of TNF and saline treated rats.

FIG 5.

SPONTANEOUS RELEASE O_2^- HILLTOP SPRAGUE–DAWLEY (BR)

B) MONTHLY VALUES



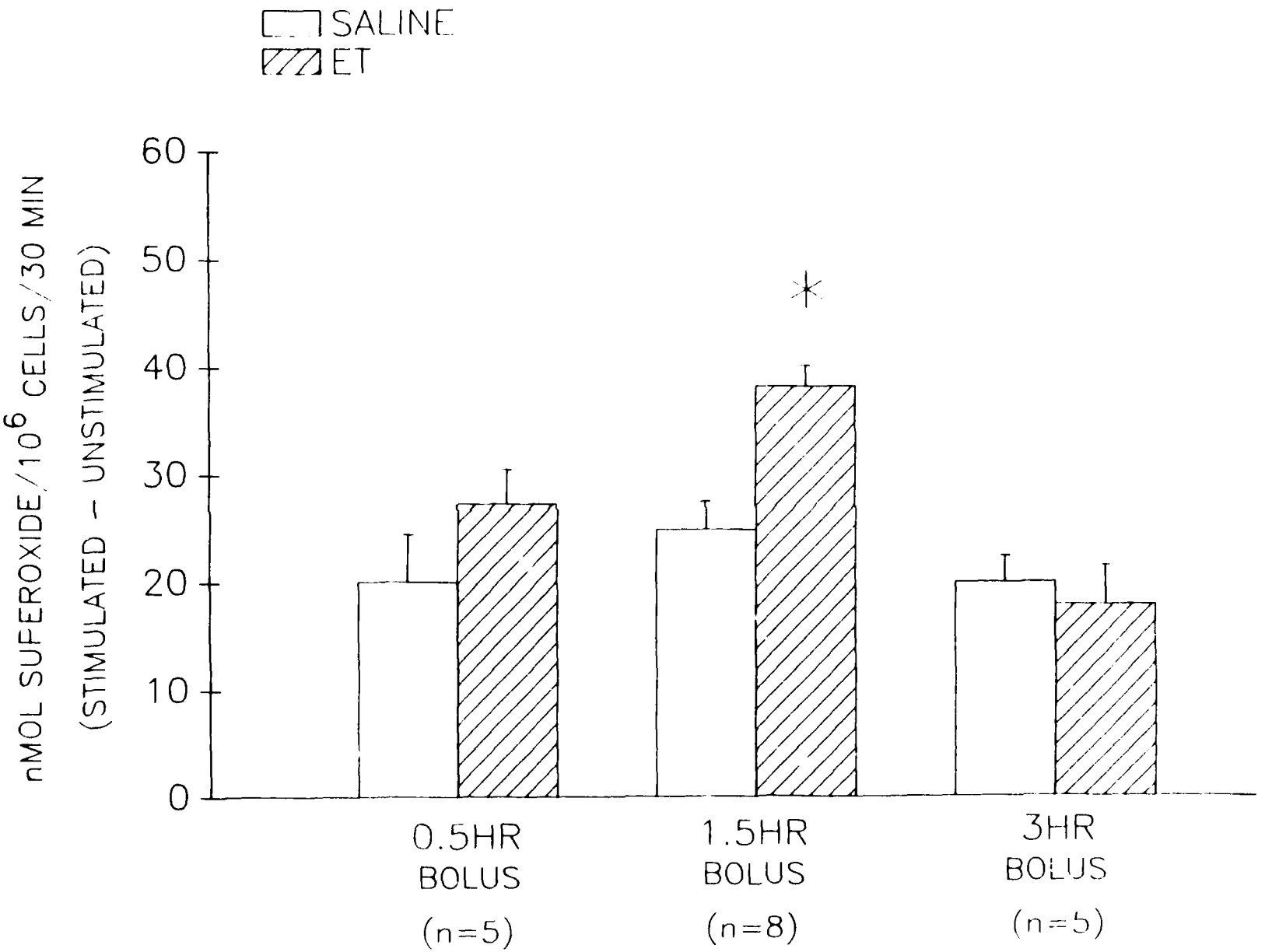


Fig. 6 Time course of OPZ-stimulated SO release by AMs of saline and ET-treated rats.

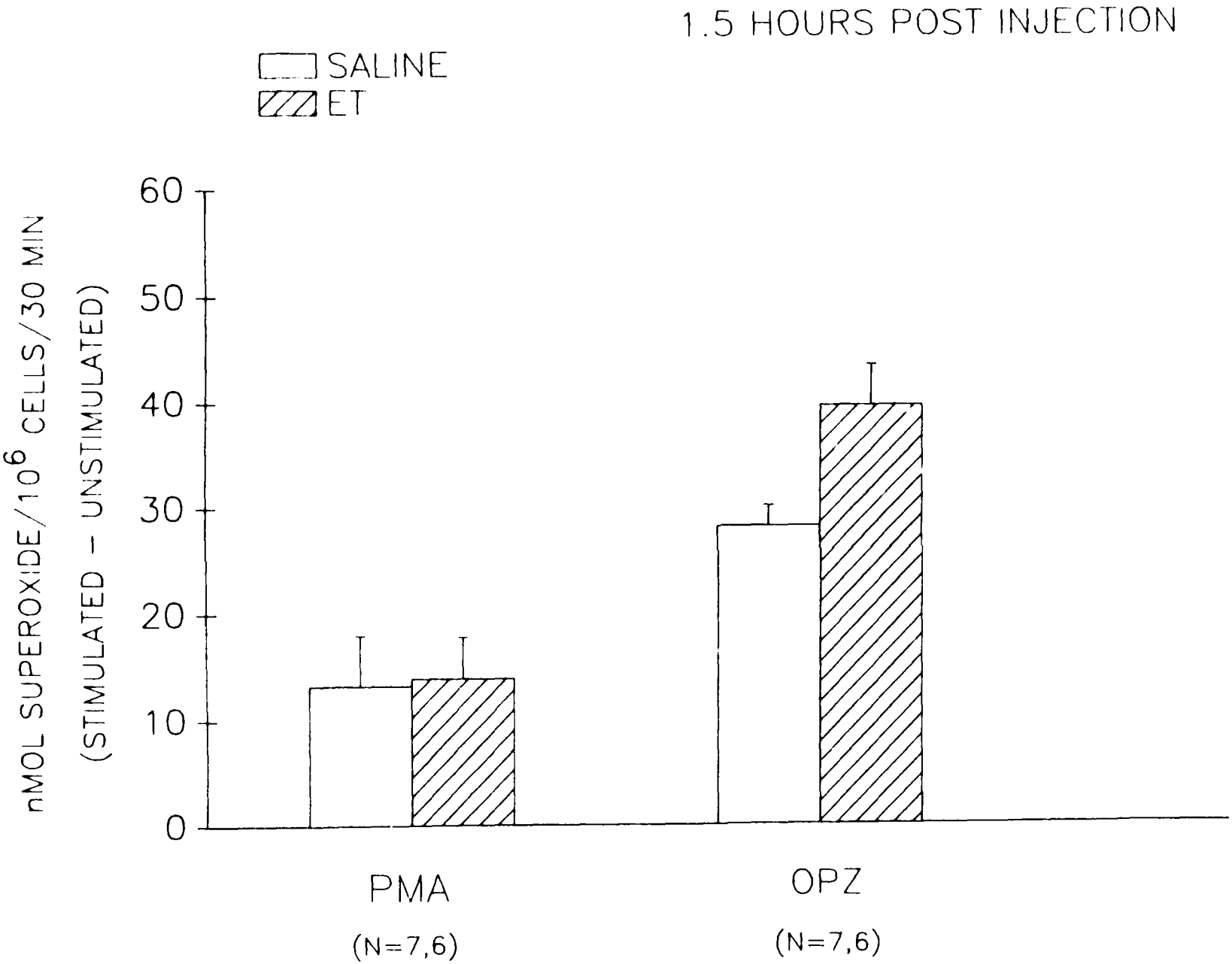


Fig. 7 OPZ- and PMA-stimulated SO release by AMs of rats 1.5h after injection of a non-lethal dose of ET (500ug/kg)

FIG. 5

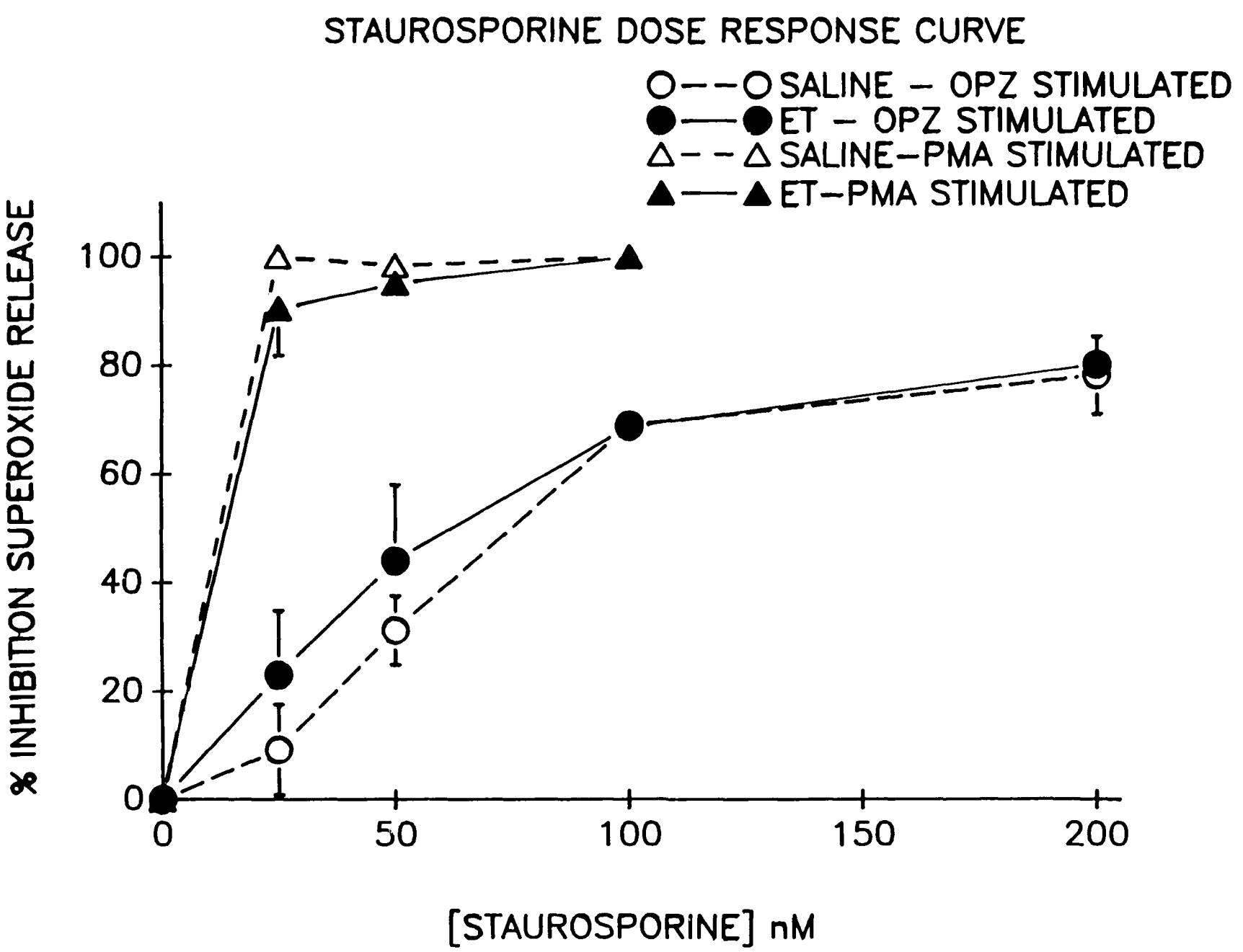

STAUROSPORINE DOSE RESPONSE CURVE
SALINE – OPZ STIMULATED
ET – OPZ STIMULATED
SALINE–PMA STIMULATED
ET–PMA STIMULATED
% INHIBITION SUPEROXIDE RELEASE
100
80
60
40
20
0
0
50
100
150
200
[STAUROSPORINE] nM

TABLE 1

Cell distribution before and after gradient separation in the 45 ml/min elutriated fraction of livers of ET-infused rats.•

Before gradient		<——— % ———>		After gradient	
K	PMN	K	PMN	K	PMN
41±1.6	52±2.9	71±4.6	21±2.4	12±3.7	84±3.7

• Means ± SEM of 4 separate experiments.

K = Kupffer cells

PMN = polymorphonuclear leukocytes

Table 2

SO release* by hepatic NP cells

Treatment and cell type	Spontaneous	PMA 10^{-8}M	OPZ 3500 μg/10^6 cells
Saline - NP	0.4±0.3	2.2±1.4	0.8±0.6
ET - NP	5.4±2.8	108.3±11.2	40.7±10.0
ET - K	0±0	49.5±10.3	15.7±4.6
ET - PMN	7.1±4.3	159±11.8	56.7±16.7

* nmoles/10^6 cells/30 min. Means ± SEM of 4 separate experiments

NP = nonparenchymal cells
(45 ml/min elutriation fraction)

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IN VIVO TUMOR NECROSIS FACTOR (TNF) ADMINISTRATION PRIMES PMA-INDUCED SUPEROXIDE (SO) PRODUCTION BY RAT ALVEOLAR MACROPHAGES. Alejandro M.S. Mayer and Judy A. Spitzer. Dept. of Physiology, LSU Med. Ctr., New Orleans, LA.

The object of this investigation was to study the effect of in vivo intravenous (IV) and intratracheal (IT) bolus injection of 6 x 10^{-5} U of human recombinant TNF administration on SO release from rat alveolar macrophages (AMs). AMs were isolated by bronchoalveolar lavage from male Sprague-Dawley rats 90 minutes after either IV or IT administration of either saline or TNF. SO production by AMs was elicited using PMA (10^{-5}-10^{-11} M) and opsonized zymosan (OZ) (500 - 3500 $\mu g/10^6$ cells) as stimuli. SO release (nmol/10^6 cells) was assayed as SOD-inhibitable reduction of ferricytochrome C and expressed as % of saline control values. AMs isolated 1.5 hours after bolus injection of TNF showed the following % increase of PMA-induced SO over saline control values: IV injection: 10^{-5} M: 180%; 10^{-6} M: 91%; 10^{-7} M: 72%; 10^{-8} M: 68%; 10^{-9} M: 57.9%; and 10^{-10} M: 10.8%; IT injection: 10^{-5} M: 244%; 10^{-6} M: 297%; 10^{-7} M: 227%; 10^{-8}:M 191% and 10^{-9} M: 818.6%. In vivo pretreatment with TNF did not prime SO release upon OZ stimulation. Thus, in vivo administration of TNF caused priming of PMA-induced SO production in AMs, implicating the possible activation of protein kinase C or some factor common to both TNF and PMA signal transduction pathways for SO release in AMs.

Supported by ONR Grant N00014-89-J-1916 (JAS). TNF was a gift from Cetus Immune Corp.

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EFFECT OF SYSTEMIC ENDOTOXIN ON SUPEROXIDE ANION RELEASE AND SIGNAL TRANSDUCTION IN RAT ALVEOLAR MACROPHAGES.

B.J.Dowty, E.Rodriguez de Turco, S. Nelson*, A.Hagar, and J.A. Spitzer.
Departments of Physiology and Pulmonary Critical Care Medicine*, LSU Medical Center, New Orleans, LA, 70112

Opsonized zymosan (OPZ)-stimulated superoxide anion release (O_2^-) and turnover of ^{32}P-phospholipids (PLs) were examined in alveolar macrophages (AMs) from rats after (1.5-3h) intravenous (i.v.) administration of saline (SAL) or a nonlethal dose of endotoxin (ET) (500ug/kg bw). OPZ stimulation resulted in increased ^{32}P-phosphatidic acid (PA) that was significantly attenuated in AMs of the ET group. OPZ stimulation was accompanied by increased ^{32}P-lysophosphatidylcholine (LPC) and a trend for decreased ^{32}P-phosphatidylcholine (PC). Basal ^{32}P-LPC was lower in AMs from the ET treatment group. These changes suggested both OPZ stimulation and ET treatment affected PLA_2-mediated mechanisms. No changes in ^{32}P-phosphatidylethanolamine were observed. A transient increase in O_2^- release following 30 min of OPZ stimulation in vitro was measured in AMs isolated 1.5 h following bolus injection of ET, that had abated by 3 h post-ET. Both mepacrine (a PLA_2 inhibitor) and staurosporine (a PKC inhibitor) inhibited OPZ-stimulated O_2^- release in a dose-dependent manner. Maximum inhibition was 90% with mepacrine and 80% with staurosporine and was not different between the two treatment groups. The data suggest that (a) an i.v. nonlethal dose of ET resulted in transient priming of AMs for increased OPZ-stimulated O_2^- release in vitro, (b) both PLA_2-and PKC-mediated mechanisms were involved in OPZ-stimulated O_2^- release and (c) ET attenuated both OPZ-stimulated accumulation of ^{32}P-PA and basal PLA_2 hydrolysis of ^{32}P-PC. The mediator of these cross-compartmental effects of ET on AMs is unknown.
Supp. by GM32654 (JAS) and ONR NOOO14-89-J-1916 (JAS).

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